JCM Accepts, published online ahead of print on 21 November 2012
J. Clin. Microbiol. doi:10.1128/JCM.02142-12

Inter-Laboratory and Inter-Study Reproducibility of a Novel Lateral-Flow Device and the Influence of Antifungal Therapy on the Detection of Invasive Pulmonary Aspergillosis

Nathan P. Wiederhold[1,2], Laura K. Najvar[1,3], Rosie Bocanegra[1,3], William R. Kirkpatrick[1,3], Thomas F. Patterson[1,3], Christopher R. Thornton[4]

[1]University of Texas Health Science Center at San Antonio, San Antonio, TX

[2]University of Texas at Austin College of Pharmacy, Austin, TX

[3]South Texas Veterans Health Care System, San Antonio, TX

[4]University of Exeter, Exeter, United Kingdom

Abstract Word Count: 248 **Manuscript Word Count:** 3508

Key Words: Invasive aspergillosis, lateral flow device, *Aspergillus fumigatus*

Corresponding Author:

Nathan P. Wiederhold, Pharm.D.

UTHSCSA

Medicine / Infectious Diseases, MC 7881

7703 Floyd Curl Drive

San Antonio, TX 78229

Phone: 210-567-8340

Email: wiederholdn@uthscsa.edu

ABSTRACT

Lateral-flow devices (LFD) have gained interest as potential point-of-care assays for the diagnosis of infectious diseases. Our objective was to evaluate the inter-laboratory and inter-study reproducibility and the effects of antifungal therapy on a LFD developed for invasive pulmonary aspergillosis (IPA) detection. An established neutropenic guinea pig model of IPA caused by *Aspergillus fumigatus* was used. At predetermined time points (1 hr, 3, 5, and 7 days post-inoculation) blood and BAL fluid were collected from infected and uninfected animals. In a separate experiment, guinea pigs were treated with posaconazole (10 mg/kg PO BID), voriconazole (10 mg/kg PO BID), liposomal amphotericin B (10 mg/kg IP QD), or caspofungin (2 mg/kg IP QD), and samples were collected on days 7 and 11. Each laboratory independently evaluated the IgG monoclonal antibody-based LFD. Galactomannan and (1→3)-β-D-glucan were also measured using commercially available kits. Good inter-laboratory agreement was observed with the LFD as 97% (32/33) of the serum and 78.8% (26/33) of the BAL samples from infected animals were in agreement. Good inter-study agreement was also observed. The serum sensitivity of each surrogate marker assay was reduced in animals treated with antifungals. In contrast, these markers remained elevated within the BAL fluids of treated animals, which was consistent with the fungal burden and histopathology results. These results demonstrate that the LFD assay is reproducible between different laboratories and studies. However, the sensitivity of this assay and other markers of IPA may be reduced within the serum in the presence of antifungal therapy.

INTRODUCTION

Invasive pulmonary aspergillosis (IPA) remains a clinically challenging opportunistic fungal infection and leads to significant morbidity and mortality in heavily immunocompromised patients (9). Despite recent advances in antifungal pharmacology and the availability of agents with improved efficacy and safety, response rates in the treatment of IPA remain suboptimal. The prompt diagnosis of this disease can have a significant impact on patient outcomes as the early diagnosis and initiation of antifungal therapy has been shown to reduce mortality in patients with invasive fungal infections including IPA (1, 3, 4). Detection of IPA relies on supporting evidence from clinical, microbiological, radiological, serological, and histopathological investigations. Rapid diagnosis of this disease has focused on the detection of surrogate markers of infection, including components of the cell wall within different biological fluids, such as the serum and bronchoalveolar lavage (BAL) fluid, and urine. Commercially available assays include those that detect galactomannan (GM) and (1→3)-β-D-glucan, which are now included in the diagnostic criteria for IPA (24). Although these tests have advanced the diagnosis of this invasive mycosis, they do have limitations, including the time required to run these assays and return the results to the clinicians, and the equipment needed to perform the assays as well as the potential for false positive reactions. In addition, the sensitivity of these assays may be reduced with antifungal exposure (10, 11, 14).

Lateral-flow technology can be used to incorporate immunochromatographic assays into simple-to-use devices for point-of-care diagnosis of infectious diseases. These lateral-flow devices (LFDs) have been used in the diagnosis of diseases caused by bacteria, fungi, toxins, and viruses, including HIV (6-8, 15, 18). We have previously

reported on the successful incorporation of an *Aspergillus*-specific murine monoclonal antibody (mAb) into a lateral-flow device (LFD). This monoclonal antibody (JF5 IgG3) binds to an extracellular glycoprotein antigen secreted constitutively during the active growth of *Aspergillus* hyphae (22). This LFD has been shown to be a sensitive and specific assay for the rapid serodiagnosis of IPA in an established guinea pig model of this invasive fungal infection (26). Furthermore, recent studies have reported preliminary findings of the potential of the LFD to diagnose IPA in haematological malignancy patients by using BAL fluids (5, 21). Here, we expand upon these initial studies by investigating LFD detection of the *Aspergillus* antigen in guinea pig BAL and serum samples simultaneously, and in comparison to commercial GM and (1→3)-β-D-glucan tests. In doing so, we (1) evaluate the inter-laboratory variability of the LFD, (2) determine the reproducibility of this assay between different studies and, (3) assess the effects of antifungal exposure on this novel assay for IPA detection.

MATERIALS AND METHODS

Isolate. *Aspergillus fumigatus* clinical isolate 293 (AF293) was grown on potato dextrose agar at 37°C for 7 days. Conidia were harvested by washing and scraping agar surfaces with 0.1% Tween 80 in sterile physiological saline, and filtering for removal of hyphal fragments. Conidia were then concentrated through centrifugation and re-suspended to give a final working concentration of ~1 x 10^8 conidia/mL, which was measured with a hemocytometer and confirmed by enumeration of colony-forming units (CFU).

92 **Animal Model.** Two days prior to infection, male Hartley guinea pigs (0.5 kg;
93 Charles River Laboratories, Wilmington, MA) were rendered immunosuppressed with
94 cyclophosphamide (250 mg/kg intraperitoneally; Mead Johnson, Princeton, NJ) and
95 cortisone acetate (250 mg/kg subcutaneously; Sigma, St. Louis, MO) . Additional doses
96 of cyclophosphamide (200 mg/kg) and cortisone acetate (250 mg/kg) were administered
97 on day 3 post-inoculation (23). Ceftazidime (100 mg/kg subcutaneously) was
98 administered daily for prevention of bacterial infections. Guinea pigs were exposed to
99 AF293 conidia at 1 x 10^8 conidia/mL for 1 hour in an aerosol chamber (17, 23). In the
100 antifungal treatment experiment guinea pigs were divided into one of five regimens
101 beginning 1 day post-inoculation (N = 8 per group): (i) control, (ii) posaconazole 10
102 mg/kg orally BID, (iii) voriconazole 10 mg/kg orally BID, (iv) liposomal amphotericin B
103 10 mg/kg intraperitoneally QD, or (v) caspofungin 2 mg/kg intraperitoneally QD.
104 Treatment was continued through day 8, and animals were monitored off therapy until
105 day 11. Uninfected immunosuppressed controls were also include in each experiment.
106 All animal procedures were approved by the Institutional Animal Care and Use
107 Committee at the University of Texas Health Science Center at San Antonio, and animals
108 were maintained in accordance with the American Association for Accreditation of
109 Laboratory Animal Care (13).
110 **Sample Collection.** Blood and BAL fluids were collected at multiple time points
111 from animals in separate studies performed consecutively in 2010 and 2011. These were
112 used to assess the inter-laboratory and inter-study reproducibility of the LFD. In these
113 studies, guinea pigs were challenged with AF293 conidia, but were not treated with
114 antifungal agents. Samples were collected from the animals 1 hour post challenge and on

115 days 3, 5, and 7. An additional study conducted in 2011-2012 assessed the effects of
116 antifungal therapy on the sensitivity of the LFD, as well as the commercial GM and
117 (1→3)-β-D-glucan assays. In this study, samples were collected on days 5, 7, and 11
118 post challenge. In all three studies, blood was collected by cardiac puncture and allowed
119 to clot, and the serum was collected following centrifugation. For BAL fluid, a catheter
120 was inserted into the trachea, and 3 mL of sterile phosphate buffered saline was instilled
121 into the lungs with a recovery volume of approximately 2 mL for each animal. The fluid
122 was then carefully removed and placed into a sterile container. Samples were also
123 collected from uninfected controls at each time point. The serum and BAL fluids were
124 stored frozen at -80°C until assayed.

125 **Lateral-Flow Device Assay.** A previously described lateral-flow device was used
126 to allow for simple and rapid diagnosis of invasive aspergillosis (22, 26). Briefly, an IgG
127 monoclonal antibody (JF5 IgG3) raised against an extracellular antigen secreted
128 constitutively during the active growth of *Aspergillus*, was immobilized to a defined
129 capture zone on a porous nitrocellulose membrane. JF5 IgG was also conjugated to
130 colloidal gold particles to serve as the detection reagent. The sample (100 µL of serum or
131 BAL fluid) was added to a release pad containing the antibody-gold conjugate, which
132 bound the target antigen, then passed along the porous membrane by capillary action and
133 bound to the antibody immobilized in the capture zone. Alternatively, processing was
134 performed by mixing 50 µL of the sample with 100 µL of 4% sodium EDTA in PBS
135 followed by heating in a boiling water bath for 3 minutes (21). The mixture was then
136 centrifuged for 5 minutes at 14,000 rpm, and 100 µL of the supernatant was added to the
137 LFD. Test results were available within 10 – 15 minutes after loading the sample. Bound

antigen-antibody-gold complex was observed as a red line with an intensity proportional to the antigen concentration, and results were recorded as weak positive (+), moderate positive (++), and strong positive (+++). Regardless of intensity, all positive test results indicate the presence of the JF5 antigen. Anti-mouse immunoglobulin immobilized to the membrane in a separate zone served as an internal control. In the absence of the *Aspergillus* antigen, no complex was formed in the zone containing solid-phase antibody, and a single internal control line was observed. This result was recorded as negative (-) for *Aspergillus* antigen.

(1→3)-β-D-Glucan and Galactomannan Assays. The (1→3)-β-D-glucan assay was performed using a commercially available kit (Fungitell, Associates of Cape Cod, East Falmouth, MA). Five microliters of each sample was transferred in duplicate to a 96-well cell culture tray and processed according to the manufacturer's instructions. The mean rate of change in optical density (405 nm) over time was measured using a microplate spectrophotometer (Synergy HT; Biotek Instruments, Winooski, VT) and unknowns were interpolated from a standard curve. Galactomannan was also measured with a commercially available kit (Platelia *Aspergillus* EIA, Bio-Rad Laboratories). Three hundred microliters of each sample was heat-treated following the addition of an EDTA acid solution. Fifty microliters of the treated supernatant was added to microwells containing conjugate and the rat monoclonal antibody EB-A2 and was allowed to incubate. The microwells were then washed and the substrate solution added, which formed a complex with the bound monoclonal antibody within the well forming a blue color. The optical density (OD) values of each sample, positive control, negative control, and cut-off control (supplied by the manufacturer) were measured using a microplate

161 spectrophotometer at 450 and 630 nm, and the galactomannan index (GMI) was
162 calculated as the OD of each sample divided by the mean cut-off of the control OD as
163 specified by the manufacturer.

164 **Fungal Burden and Pulmonary Histopathology.** Tissue fungal burden was
165 measured by enumeration of CFUs. Lungs from each animal were weighed, placed into
166 sterile saline containing gentamicin and chloramphenicol, homogenized, and the number
167 of CFU/gram of lung tissue was determined as previously described (16, 23). In addition
168 to serum and BAL fluid, lungs were collected for histopathology. This was done to
169 compare the results of the various surrogate markers (LFD, (1→3)-β-D-glucan and
170 galactomannan) in the serum and BAL fluid with the extent of invasive disease within the
171 lungs in animals treated with antifungal agents. Portions of the lungs at the different time
172 points were placed into 10% volume/volume neutral buffered formaldehyde. The lungs
173 were then processed and embedded into paraffin wax. Sections of embedded tissue were
174 then stained with Gomori methamine silver (GMS) stain in order to visualize the fungal
175 elements within the lungs.

176 **Data Analysis.** Two separate laboratories, each blinded to the results of the other,
177 performed the lateral-flow assay independently. Differences in the number of positive
178 samples for the same animals per time point between the laboratories and different
179 surrogate marker assays were assessed for significance by Fisher's exact test, and a p-
180 value of < 0.05 was considered statistically significant. The overall specificity of each
181 assay was also measured in uninfected controls. All statistical tests were performed using
182 Prism 5.0 (GraphPad Software, Inc.).
183

184 **RESULTS**

185 **Inter-laboratory Reproducibility.** To compare the inter-laboratory
186 reproducibility of the LFD, two laboratories (Exeter and UT Health Science Center at San
187 Antonio) independently evaluated the samples collected from the same animals in the
188 2011 study, by applying 100 µL of serum to the LFD and recording the results 10 - 15
189 minutes later. As shown in Tables 1 and 2, there was good agreement between the
190 laboratories (90 - 100%) at each time point in the samples collected from infected
191 animals as well as those from uninfected controls. When the samples were processed (50
192 µL of serum plus 100 µL 4% EDTA in PBS plus heating), similar results were observed
193 compared to unprocessed serum directly applied to the LFD. However, the antigen
194 appeared to be detected earlier in processed BAL samples (6/10 positive for one
195 laboratory and 10/10 positive from the second laboratory on day 3 post-inoculation)
196 compared to those that were applied directly (2/10 positive on day 3). While the
197 processing of the samples did increase the early sensitivity of the LFD by brightening the
198 background of the device, it did not reduce the specificity, as all BAL samples from the
199 uninfected group were negative. These results suggest that serum samples may be
200 directly applied to the LFD while further processing may improve the ability of this
201 device to detect the *Aspergillus* antigen within BAL fluid.

202 **Inter-study Reproducibility.** We also evaluated the reproducibility of the LFD
203 for the detection of IPA by comparing the results for the 2010 and 2011 studies. The
204 results for the LFD, (1→3)-β-D-glucan, and galactomannan assays in serum and BAL
205 samples collected in the consecutive studies are shown in Table 3. Overall, the results
206 from the two studies conducted with this model were comparable, both for serum and for

BAL fluids. There were fewer positive samples with the LFD in the serum earlier in the course of infection in the 2011 study compared to the one conducted previously, however, these differences were not significant. In addition, these results were similar to the clinically available assays for (1→3)-β-D-glucan and galactomannan as there were no significant differences in the number of samples that were positive between the different assays at each time point. Although the cumulative results suggest that the LFD became positive within the serum earlier in the course of the model compared to the (1→3)-β-D-glucan assay (40% vs. 5.7% positive rate in infected animals on day 3; $p < 0.01$), this outcome was not consistent between studies. There were also significantly fewer positive tests in the BAL fluid collected from uninfected controls with the LFD compared to the (1→3)-β-D-glucan and galactomannan assays ($p < 0.01$). These data demonstrate that the LFD assay is reproducible with consistent and specific results within the serum and BAL fluid between different experiments.

Influence of Antifungal Therapy on the LFD, (1→3)-β-D-Glucan, and Galactomannan Assays. We also evaluated whether exposure to antifungal agents would affect the ability the LFD, (1→3)-β-D-glucan, and galactomannan assays to detect their respective surrogate markers of IPA both within the serum and BAL fluids. Both (1→3)-β-D-glucan concentrations and the GM index were lower within the serum of animals that received antifungal therapy compared to untreated controls (Figure 1). This was especially evident with posaconazole, voriconazole, and liposomal amphotericin B as these surrogate markers were below the threshold of positivity ((1→3)-β-D-glucan 80 pg/mL and serum GM index 0.5) in the majority of guinea pigs at day 7 (1 positive result for both the (1→3)-β-D-glucan and galactomannan assays in 19 tested samples from

infected guinea pigs). In contrast, the two markers within the serum were above these thresholds in most animals treated with caspofungin. The results of the LFD were also affected by antifungal exposure as most serum samples were negative in the animals that received antifungal therapy at this time point, including those samples taken from animals that received caspofungin (4 positive results from 22 tested samples). In contrast, the majority of samples collected from untreated infected controls were positive with each assay. Similar results were also observed on day 11.

The results from the serum of animals that received antifungal therapy are in contrast to what was observed within the BAL fluid and the fungal burden data. As shown in Figure 1, the majority of BAL samples remained positive with each assay despite exposure to antifungal agents. For the galactomannan assay in the BAL samples, a GM index of $\geq$ 1.0 was considered the threshold value for positivity. The results for all three diagnostic tests in the BAL fluid are in agreement with the pulmonary fungal burden data as the CFU counts within the lungs of the animals that received therapy (mean range 3.26 - 3.95 $\log_{10}$ CFU/g) did not differ significantly from the untreated controls (3.84 $\log_{10}$ CFU/g), regardless of the antifungal agent that was used (Figure 2). Invasive hyphae were also seen in the infected controls as well as those treated with liposomal amphotericin B or caspofungin (representative histopathology sections are shown in Figure 2). Although lung damage was observed in animals treated with posaconazole, no hyphae are observed. For voriconazole, no hyphae were visible and little damage was found within these sections. It is unclear from these data if the histopathology results are due to sampling bias for the posaconazole and voriconazole, or if hyphae were absent but the lung fungal burden and surrogate marker results were

caused by the presence of germlings or fragmented hyphae secondary to antifungal exposure. *Aspergillus* colonies were not detected within the lungs of uninfected controls. This is consistent with the results of the galactomannan and LFD assays, which were negative within the BAL fluid. However, the (1→3)-β-D-glucan assay was positive in the majority of BAL samples collected from uninfected controls.

DISCUSSION

The prompt diagnosis of invasive pulmonary aspergillosis can have significant impacts on patient outcomes. Early diagnosis and initiation of antifungal therapy has been shown to reduce mortality in patients with invasive fungal infections including IPA (1, 3, 4). In the absence of a single 'gold standard' test, the diagnosis of this opportunistic mycosis relies on clinical data and microbiology and histopathology where feasible. Rapid diagnosis of this disease has focused on the detection of surrogate markers of infection within the serum and BAL fluid. Two commercially available tests for measuring surrogate markers of invasive aspergillosis include the Fungitell (1→3)-β-D-glucan and Platelia *Aspergillus* galactomannan assays. The Fungitell test is a chromogenic assay based on the activation of the horseshoe crab coagulation cascade by (1→3)-β-D-glucan, and uses amebocyte enzymes from *Limulus polyphemus* (2). When this fungal cell wall component is present, the coagulation cascade is triggered and ultimately results in the release of a chromogenic peptide that can be measured using a microplate spectrophotometer. The Platelia *Aspergillus* ELISA kit (Bio-Rad) uses a rat monoclonal antibody (EB-A2) directed against the immunodominant epitope within galactomannan, a cell wall component of *Aspergillus* released during growth (12, 19, 20). Both assays have improved the diagnosis of IPA and are part of the Infectious Disease

Society of America guidelines for this invasive fungal infection (24). However, these assays do have limitations, including the need for dedicated time and equipment to perform the tests, which may increase the time it takes for the results to reach clinicians and delay the start of appropriate antifungal therapy. Thus, there is a need for the development of point-of-care devices, such as LFDs, that allow for the rapid, reliable, and routine testing of samples from patients at high risk for IPA.

We have previously reported the development of a LFD that utilizes an IgG3 mAb, JF5, that binds to an extracellular antigen secreted constitutively during the active growth of *Aspergillus*. The JF5 monoclonal antibody reacts strongly with antigens from *Aspergillus* species, but does not react with other pathogenic fungi, including *Candida* species, *Cryptococcus neoformans*, *Fusarium* species, *Scedosporium prolificans*, *Pseudallescheria boydii*, and the causative agents of mucormycosis (22). Benefits of this investigational assay include the short time needed to obtain results (10 - 15 minutes) and the minimal processing of samples. In addition, the LFD became positive early during the course of infection in a previous study with this guinea pig model of IPA, while maintaining specificity with negative results in uninfected controls (26). However, in the present study, the earlier detection of the antigen with the LFD was not consistent for all experiments. This is likely due to the inherent variability of animal responses to infection and associated production of diagnostic markers in each set of experiments, and further work is needed to determine the consistency of the LFD in detecting disease earlier than surrogate marker assays.

The results of the current study are encouraging as they demonstrated that the results of this LFD are reproducible between laboratories and different studies. Little

299 variance was observed between the two laboratories, and the cumulative results of this
300 study and our previous work show that this assay is comparable to the clinically available
301 assays for the detection of (1→3)-β-D-glucan and galactomannan. We also demonstrate
302 that the sensitivity of the LFD within BAL fluid may be improved with minimal sample
303 processing without compromising its specificity as there were significantly fewer positive
304 samples with this test from uninfected guinea pigs compared to the other assays.
305 However, the sensitivity of the LFD, along with the (1→3)-β-D-glucan and
306 galactomannan tests, was affected by antifungal exposure. The ability of each diagnostic
307 assay to detect the surrogate markers of disease within the serum was reduced in animals
308 exposed to antifungals. These results are consistent with previous reports of reduced
309 sensitivity of the (1→3)-β-D-glucan and galactomannan assays in patients with IPA who
310 received antifungal therapy (10, 14). In our study, this most likely represents the
311 suppression of disease dissemination and reduction of the *Aspergillus* antigens within the
312 sera to levels below the limit of detection for each surrogate marker assay, but not
313 clearance of the disease from the lungs as the pulmonary fungal burden remained
314 elevated even in animals that received antifungal therapy. In addition, positive results
315 were observed within the BAL samples for each diagnostic assay. We have previously
316 demonstrated that commonly used antifungals and antibiotics do not result in false
317 positive or false negative results with the LFD as clinically relevant concentrations (25).
318 Although the results of this study are promising, one limitation is that this LFD
319 has been evaluated in only one animal model of IPA. Thus, there is a need for additional
320 studies with this assay. In addition, positive results occurred with each surrogate marker
321 assay in some samples collected from uninfected controls. As these guinea pigs were not

exposed to *A. fumigatus* conidia via the aerosol chamber, were separated from the infected control group throughout the course of these experiments, and fungal colonies were not detected within the lungs, this most likely represents environmental exposure to *Aspergillus* antigens. The sterile food and bedding used in the housing of these animals has tested positive for each surrogate marker assay used in these studies and may be a source of these antigens (data not shown). Finally, while the results with a small number of clinical samples have been promising (22), a larger number of samples from patients with proven or probable IPA need to be evaluated in order to confirm the potential clinical utility of this LFD for the diagnosis of this infectious disease.

FUNDING AND ACKNOWLEDGEMENTS

The authors would like to thank Marcos Olivo with his assistance with the animal models. This work was supported by contracts from the National Institutes of Health / National Institute of Allergy and Infectious Diseases **(**Contract Nos. N01-AI-30041, and HHSN272201000038I/HHSN27200002 - Task Order A05) to T.F. Patterson, and NIH/NIAID grant R21AI085393 to N.P. Wiederhold and C.R. Thornton.

TRANSPARENCY DECLARATION

NPW has received research support from Pfizer, Schering-Plough, Merck, Basilea, and Astellas, and has served on advisory boards for Merck, Astellas, Toyama, and Viamet. TFP has received research grants to UT Health Science Center San Antonio from Astellas, Basilea, Merck, and Pfizer, and as a consultant for Astellas, Basilea, Merck, Pfizer, and Toyama. CRT has received support from Pfizer. All other authors: no conflicts.

REFERENCES

1. **Caillot, D., O. Casasnovas, A. Bernard, J. F. Couaillier, C. Durand, B. Cuisenier, E. Solary, F. Piard, T. Petrella, A. Bonnin, G. Couillault, M. Dumas, and H. Guy.** 1997. Improved management of invasive pulmonary aspergillosis in neutropenic patients using early thoracic computed tomographic scan and surgery. J. Clin. Oncol. 15:139-147.
2. **Fungitell.** 2008. Assay for (1,3)-beta-D-glucan in serum. Associates of Cape Cod Incorporated, E. Falmouth, MA.
3. **Garey, K. W., M. Rege, M. P. Pai, D. E. Mingo, K. J. Suda, R. S. Turpin, and D. T. Bearden.** 2006. Time to initiation of fluconazole therapy impacts mortality in patients with candidemia: a multi-institutional study. Clin. Infect. Dis. 43:25-31.
4. **Greene, R. E., H. T. Schlamm, J. W. Oestmann, P. Stark, C. Durand, O. Lortholary, J. R. Wingard, R. Herbrecht, P. Ribaud, T. F. Patterson, P. F. Troke, D. W. Denning, J. E. Bennett, B. E. de Pauw, and R. H. Rubin.** 2007. Imaging findings in acute invasive pulmonary aspergillosis: clinical significance of the halo sign. Clin. Infect. Dis. 44:373-379.
5. **Hoenigl, M., C. Koidl, W. Duettmann, K. Seeber, J. Wagner, W. Buzina, A. Wolfler, R. B. Raggam, C. R. Thornton, and R. Krause.** 2012. Bronchoalveolar lavage lateral-flow device test for invasive pulmonary aspergillosis diagnosis in haematological malignancy and solid organ transplant patients. J. Infect. 65:588-591.
6. **Iweala, O. I.** 2004. HIV diagnostic tests: an overview. Contraception 70:141-147.

7. **Jarvis, J. N., A. Percival, S. Bauman, J. Pelfrey, G. Meintjes, G. N. Williams, N. Longley, T. S. Harrison, and T. R. Kozel.** 2011. Evaluation of a novel point-of-care cryptococcal antigen test on serum, plasma, and urine from patients with HIV-associated cryptococcal meningitis. Clin. Infect. Dis. 53:1019-1023.

8. **Ketema, F., C. Zeh, D. C. Edelman, R. Saville, and N. T. Constantine.** 2001. Assessment of the performance of a rapid, lateral flow assay for the detection of antibodies to HIV. J. Acquir. Immune Defic. Syndr. 27:63-70.

9. **Lin, S. J., J. Schranz, and S. M. Teutsch.** 2001. Aspergillosis case-fatality rate: systematic review of the literature. Clin. Infect. Dis. 32:358-366.

10. **Marr, K. A., M. Laverdiere, A. Gugel, and W. Leisenring.** 2005. Antifungal therapy decreases sensitivity of the Aspergillus galactomannan enzyme immunoassay. Clin. Infect. Dis. 40:1762-1769.

11. **McCulloch, E., G. Ramage, R. Rajendran, D. F. Lappin, B. Jones, P. Warn, R. Shrief, W. R. Kirkpatrick, T. F. Patterson, and C. Williams.** 2012. Antifungal treatment affects the laboratory diagnosis of invasive aspergillosis. J. Clin. Pathol. 65:83-86.

12. **Morelle, W., M. Bernard, J. P. Debeaupuis, M. Buitrago, M. Tabouret, and J. P. Latge.** 2005. Galactomannoproteins of Aspergillus fumigatus. Eukaryot. Cell 4:1308-1316.

13. **NAS.** 1996. Guide for the care and use of laboratory animals. National Academy Press, Washington, D.C.

14. **Senn, L., J. O. Robinson, S. Schmidt, M. Knaup, N. Asahi, S. Satomura, S. Matsuura, B. Duvoisin, J. Bille, T. Calandra, and O. Marchetti.** 2008. 1,3-

Beta-D-glucan antigenemia for early diagnosis of invasive fungal infections in neutropenic patients with acute leukemia. Clin. Infect. Dis. 46:878-885.

15. **Sharma, S. K., B. S. Eblen, R. L. Bull, D. H. Burr, and R. C. Whiting.** 2005. Evaluation of lateral-flow Clostridium botulinum neurotoxin detection kits for food analysis. Appl. Environ. Microbiol. 71:3935-3941.

16. **Sheppard, D. C., J. R. Graybill, L. K. Najvar, L. Y. Chiang, T. Doedt, W. R. Kirkpatrick, R. Bocanegra, A. C. Vallor, T. F. Patterson, and S. G. Filler.** 2006. Standardization of an experimental murine model of invasive pulmonary aspergillosis. Antimicrob. Agents Chemother. 50:3501-3503.

17. **Sheppard, D. C., G. Rieg, L. Y. Chiang, S. G. Filler, J. E. Edwards, Jr., and A. S. Ibrahim.** 2004. Novel inhalational murine model of invasive pulmonary aspergillosis. Antimicrob. Agents Chemother. 48:1908-1911.

18. **Smits, H. L., C. K. Eapen, S. Sugathan, M. Kuriakose, M. H. Gasem, C. Yersin, D. Sasaki, B. Pujianto, M. Vestering, T. H. Abdoel, and G. C. Gussenhoven.** 2001. Lateral-flow assay for rapid serodiagnosis of human leptospirosis. Clin. Diagn. Lab. Immunol. 8:166-169.

19. **Stynen, D., A. Goris, J. Sarfati, and J. P. Latge.** 1995. A new sensitive sandwich enzyme-linked immunosorbent assay to detect galactofuran in patients with invasive aspergillosis. J. Clin. Microbiol. 33:497-500.

20. **Stynen, D., J. Sarfati, A. Goris, M. C. Prevost, M. Lesourd, H. Kamphuis, V. Darras, and J. P. Latge.** 1992. Rat monoclonal antibodies against Aspergillus galactomannan. Infect. Immun. 60:2237-2245.

412 **21.** **Thornton, C., G. Johnson, and S. Agrawal.** 2012. Detection of invasive
413 pulmonary aspergillosis in haematological malignancy patients by using lateral-
414 flow technology. J. Vis. Exp.:e3721.
415 **22.** **Thornton, C. R.** 2008. Development of an immunochromatographic lateral-flow
416 device for rapid serodiagnosis of invasive aspergillosis. Clin. Vaccine Immunol.
417 15:1095-1105.
418 **23.** **Vallor, A. C., W. R. Kirkpatrick, L. K. Najvar, R. Bocanegra, M. C. Kinney,**
419 **A. W. Fothergill, M. L. Herrera, B. L. Wickes, J. R. Graybill, and T. F.**
420 **Patterson.** 2008. Assessment of Aspergillus fumigatus burden in pulmonary
421 tissue of guinea pigs by quantitative PCR, galactomannan enzyme immunoassay,
422 and quantitative culture. Antimicrob. Agents Chemother. 52:2593-2598.
423 **24.** **Walsh, T. J., E. J. Anaissie, D. W. Denning, R. Herbrecht, D. P. Kontoyiannis,**
424 **K. A. Marr, V. A. Morrison, B. H. Segal, W. J. Steinbach, D. A. Stevens, J. A.**
425 **van Burik, J. R. Wingard, and T. F. Patterson.** 2008. Treatment of
426 aspergillosis: clinical practice guidelines of the Infectious Diseases Society of
427 America. Clin. Infect. Dis. 46:327-360.
428 **25.** **Wiederhold, N. P., and C. R. Thornton.** 2011. Lack of cross-reactivity and
429 interference of commonly used intravenous antimicrobials with a lateral-flow
430 device for the diagnosis of invasive aspergillosis, abstr M-1538. 51st Intersci.
431 Conf. Antimicrob. Agents Chemother., Chicago, IL.
432 **26.** **Wiederhold, N. P., C. R. Thornton, L. K. Najvar, W. R. Kirkpatrick, R.**
433 **Bocanegra, and T. F. Patterson.** 2009. Comparison of lateral flow technology

434 and galactomannan and (1->3)-beta-D-glucan assays for detection of invasive
435 pulmonary aspergillosis. Clin. Vaccine Immunol. 16:1844-1846.

436
437
438
439
440
441
442
443
444
445
446
447
448
449
450
451
452
453
454
455
456

457 **FIGURE LEGENDS**

458 **Figure 1.** (1→3)-β-D-glucan, galactomannan, and lateral-flow device (LFD) results
459 using the serum and bronchoalveolar lavage (BAL) fluid from guinea pigs with invasive
460 aspergillosis that were exposed to antifungal agents. Antifungal therapy with
461 posaconazole (PSC 10 mg/kg PO BID), voriconazole (VRC, 10 mg/kg PO BID),
462 liposomal amphotericin B (LAMB, 10 mg/kg IP QD), or caspofungin (CFG, 2 mg/kg IP
463 QD) began one day after aerosol inoculation and continued through day 8. Serum
464 samples were collected on day 7 and day 11 in animals that survived to the study
465 endpoint, and BAL samples were collected on day 11.
466
467 **Figure 2.** Pulmonary fungal burden (A) and representative histopathology sections from
468 guinea pigs that received antifungal therapy (B). Antifungal therapy with posaconazole
469 (PSC 10 mg/kg PO BID), voriconazole (VRC, 10 mg/kg PO BID), liposomal
470 amphotericin B (LAMB, 10 mg/kg IP QD), or caspofungin (CFG, 2 mg/kg IP QD) began
471 one day after aerosol inoculation and continued through day 8. Lungs were collected on
472 day 11 or when the animals succumbed to infection, and the line depicts the median $\log_{10}$
473 CFU/g. For histopathology lungs sections were stained with GMS and viewed at 200X
474 magnification by light microscopy.
475
476
477
478

Table 1. Inter-laboratory comparison of the lateral-flow device (LFD) in serum from the 2011 study. The unprocessed samples (100 µL) were applied directly to the LFD and the results were read 10 - 15 minutes later. For the processed samples, 50 µL of the serum were mixed with 100 µL of 4% sodium EDTA in PBS, followed by heating for 3 minutes in a boiling water bath, and centrifugation. The supernatant (100 µL) was then applied to the LFD. Agreement between labs for unprocessed serum samples is calculated between individual animals.

Time Point	Laboratory 1 (Unprocessed Serum)	Laboratory 2 (Unprocessed Serum)	Serum LFD Agreement Between Labs	Laboratory 2 (Processed Serum)
Serum samples from infected guinea pigs				
1 hour	0/3	0/3	3/3 (100%)	0/3
Day 3	0/10	0/10	10/10 (100%)	2/10
Day 5	4/10	5/10	9/10 (90%)	7/10
Day 7	7/10	7/10	10/10 (100%)	8/10
Aggregate agreement between laboratories			32/33 (97%)	---
Serum samples from uninfected guinea pigs				
1 hour	0/3	0/3	3/3 (100%)	0/3
Day 3	0/3	0/3	3/3 (100%)	0/3
Day 5	0/3	0/3	3/3 (100%)	1/3
Day 7	2/3	2/3	3/3 (100%)	2/3
Aggregate agreement between laboratories			12/12 (100%)	---

Table 2. Inter-laboratory comparison of the lateral-flow device (LFD) in bronchoalveolar lavage (BAL) fluids from the 2011 study. The unprocessed samples (100 µL) were applied directly to the LFD and the results were read 10 - 15 minutes later. For the processed samples, 50 µL of the BAL fluid were mixed with 100 µL of 4% sodium EDTA in PBS, followed by heating for 3 minutes in a boiling water bath, and centrifugation. The supernatant (100 µL) was then applied to the LFD. Agreement between labs for processed BAL samples is calculated between individual animals.

Time Point	Laboratory 1 (Processed BAL)	Laboratory 2 (Processed BAL)	BAL LFD Agreement Between Labs	Laboratory 1 (Unprocessed BAL)
BAL samples from infected guinea pigs				
1 hour	0/3	0/3	3/3 (100%)	0/3
Day 3	6/10	10/10	6/10 (60%)	2/10
Day 5	10/10	10/10	10/10 (100%)	10/10
Day 7	7/10	10/10	7/10 (70%)	9/10
Aggregate agreement between laboratories			26/33 (78.8%)	---
BAL samples from uninfected guinea pigs				
1 hour	0/3	0/3	3/3 (100%)	0/3
Day 3	0/3	0/3	3/3 (100%)	0/3
Day 5	0/3	0/3	3/3 (100%)	0/3
Day 7	0/3	0/3	3/3 (100%)	0/3
Aggregate agreement between laboratories			12/12 (100%)	---

496 **Table 3.** Comparison of the lateral-flow device (LFD), (1→3)-β-D-glucan, and galactomannan assays in serum and bronchoalveolar
497 lavage (BAL) fluids for the 2010 and 2011 studies, as well as the cumulative results for each surrogate marker. Results are shown as
498 number of positive results/number of samples tested.

Study	2010			2011			Cumulative Results		
Serum Samples									
Assay	LFD	β-glucan	GM	LFD	β-glucan	GM	LFD	β-glucan	GM
1 hr	0/5	0/5	1/5	0/3	0/2	0/3	0/8 (0%)	0/7 (0%)	1/8 (12.5%)
Day 3	12/25	0/25	1/25	2/10	2/10	5/10	14/35 (40%)	2/35 (5.7%)	6/35 (17.1%)
Day 5	14/17	4/17	10/17	5/10	4/10	7/10	19/27 (70.4%)	8/27 (29.6%)	17/27 (70%)
Day 7	6/6	6/6	6/6	7/10	7/10	8/10	13/16 (81.2%)	13/16 (81.2%)	14/16 (87.5%)
Uninfected	0/10	2/10	0/10	2/12	2/10	4/12	2/22 (9.1%)	4/20 (20%)	4/22 (18.2%)
BAL Samples									
Assay	LFD	β-glucan	GM	LFD	β-glucan	GM	LFD	β-glucan	GM
1 hr	0/15	4/10	0/10	0/3	0/2	0/3	0/18 (0%)	4/12 (33%)	0/13 (0%)
Day 3	10/15	10/10	8/10	10/10	10/10	10/10	20/25 (80%)	20/20 (100%)	18/20 (90%)
Day 5	12/15	10/10	10/10	10/10	10/10	10/10	22/25 (88%)	20/20 (100%)	20/20 (100%)
Day 7	14/14	10/10	10/10	10/10	10/10	10/10	24/24 (100%)	20/20 (100%)	20/20 (100%)
Uninfected	0/21	7/16	5/16	0/12	2/10	1/12	0/33 (0%)	9/26 (34.6%)	6/28 (21%)

499